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Hemolytic Activity of Five Different Calcium Silicates

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Mineral characteristics and the *in vitro* hemolytic activity of three synthetic and two natural calcium silicates (CaSi) are compared. Hemolysis is higher for the synthetic compounds than for the natural ones. The difference is accentuated by weak ultrasonication of the minerals. No variation was observed within the two groups, including both acicular and fibrous forms. Calcium was released from the minerals during storage in Tris-buffered saline. At the same time, hemolysis decreased, and crystallographic alterations occurred in the leached minerals. Treatment of the CaSi with calcium chelators (EGTA and EDTA) did not change hemolytic activity. An increase was observed when 30 *mM* calcium was added. Hemolysis is related to specific surface areas and the crystalline structure of the minerals. Calcium may also be a contributing factor.

Introduction

Calcium silicates (CaSi) are used in various industrial products: ceramics, insulation, fillers and as a substitute for asbestos (*1*). The natural wollastonite ($CaSiO_3$) is exploited mainly in the U.S., Mexico and Finland. Synthetic varieties are also available commercially. They are produced by hydrothermal reaction between quartz and calcite. Both categories include acicular and fibrous forms. Significant *in vitro* hemolytic activity has been shown for wollastonite (*2-4*) and for other CaSi compounds (*5*). A cytotoxic effect of one synthetic CaSi has recently been demonstrated in a fibroblast cell line (*5*), and biological reactivity *in vivo* has been shown following intratracheal instillation in rats (*6*).

The objective of the present study is to compare the hemolytic activity of natural and synthetic CaSi, including both acicular and fibrous forms, and to investigate mechanisms of hemolysis related to mineralogical characteristics of the dust samples.

Material and Methods

Calcium Silicate Minerals (CaSi)

Five CaSi minerals were used: CaSi A, fibrous natural wollastonite (respirable samples of American wollastonite prepared and kindly donated by the MRC Pneumoconiosis Unit, Penarth, UK); CaSi B, natural wollastonite with few fibers (donated by Partek OY, Finland); CaSi C, nonfibrous synthetic β-wollastonite (donated by YTONG AB, Sweden); CaSi D, nonfibrous (platy) synthetic tobermorite (donated by YTONG AB, Sweden); CaSi E, fibrous synthetic tobermorite (obtained as pipe line insulation material from an oil exploitation installation in the North Sea).

UICC Chrysotile B (donated by the MRC Pneumoconiosis Unit, Penarth, UK) was included in the study as a positive control, and anatase (TiO_2 supplied by Kronos-Titan Ltd. Norway) was used as a negative control. A sample of respirable α-SiO_2 was also used for comparative studies.

Preparation and Characterization of Respirable Samples

A dust cloud was created in a closed chamber by a Timbrell dust feeder for the fibrous samples and a Wright dust feeder for the nonfibrous samples. Respirable samples were then collected by a Hexhlet dust sampler.

A scanning electron microscope (SEM) (Jeol JSM-35) was used for the morphological studies, and a Philips X-ray diffractometer equipped with a graphite crystal monochromator was used for the crystallographic investigations.

The test tubes with mineral dust suspensions were always vigorously shaken by hand before the red blood cells were added. The effect of ultrasonication of the dust suspensions for 5 and 10 min before addition of red blood cells also was examined.

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Determination of specific surface area was done by the gas adsorption technique (7).

Measurement of Hemolysis

Variation in hemolysis due to the test tube shape was observed. Therefore, 10-mL plastic test tubes of the same shape were always used.

A 5-mL portion of human blood was collected in 1.5 mL Na-citrate glucose solution (sodium citrate 2.2%, citric acid 8%, glucose 2.23%) for each experiment. After centrifugation, plasma and the buffy coat were removed and red blood cells (RBC) washed three times with Tris-buffered saline (25 *mM* Tris, 312 *mM* NaCl) pH 7.3-7.4. Blood from the same donor was used throughout the experiments.

For the investigations, a 1% RBC suspension was prepared by adding a 3.5 mL dust suspension to 1.5 mL of a stock RBC suspension. The mixture was carefully shaken and incubated in a shaking waterbath at a temperature of 37°C and at 60 vibrations/min. After centrifugation the content of hemoglobin in the supernatant was determined by adding 2 mL of DADE HiCN reagent to 1 mL of the supernatant. The cyanmethemoglobin thus formed was determined by a Vitatron DCP spectrophotometer at 540 nm.

In all experiments a fragility control was included, i.e., hemolysis of RBC without dust. The hemolytic activity was determined as the mean of two or three parallels and compared to 100% hemolysis caused by adding one drop of Triton X-100 to 5 mL of the 1% RBC suspension. The same values for 100% hemolysis could be obtained by adding 3.5 mL of water to 1.5 mL of the stock RBC suspension. During the experimental period of 3 months, there was a 15-20% loss in the hemolytic activity of the positive control UICC chrysotile B which initially was higher than hemolysis by water or by Triton X-100. Fragility control values were subtracted before calculation of the relative hemolysis.

For practical purposes it is recommended that the amount of dust used should induce 50% hemolysis or less (*8, 9*). If higher dust concentrations are used, hemoglobin and cell fragments will adsorb to the dust particles, which prevents a true reading of the degree of hemolysis. In our studies three concentrations of dust were used, and linearity was maintained even for the values exceeding 50% hemolysis. In the experiments a concentration (0.5 mg/mL) giving hemolysis generally less than 50%, was chosen.

The morphology of RBC was examined by collecting a 0.5 mL sample of the exposed cells 5 min and 60 min after the start of incubation. The samples were fixed in glutaraldehyde, then postfixed in osmium tetroxide, followed by dehydration and critical point drying before gold coating and SEM examination.

Leaching Experiments

One natural (CaSi B) and one synthetic (CaSi D) CaSi (0.5 mg/mL) were suspended in buffered saline at 37°C for 20, 40, 80, 120 and 240 min, and further for 1, 2, 3, 5, 8, 16 and 32 days. Each sample was centrifuged at 1000*g* for 20 min. The amount of calcium leached out from the dust particles was determined in the supernatant by atomic absorption spectrometry while the remaining minerals were examined by SEM and X-ray diffractometry.

Effect of EDTA, EGTA and Calcium

At 15 min before the incubation, 10 μM Tris/EDTA (ethylendiaminetetraacetic acid) buffer at pH 6.2 or pH 7.2 was added to the buffered saline containing the dust particles. The natural CaSi B and all the synthetic compounds were also treated for 24 hr with a Tris/EGTA (ethyleneglycol bis(aminoethyl ether)-*N, N*-tetraacetic acid) (2, 4, 8, 16 and 32 *mM*) and the hemolytic activity recorded.

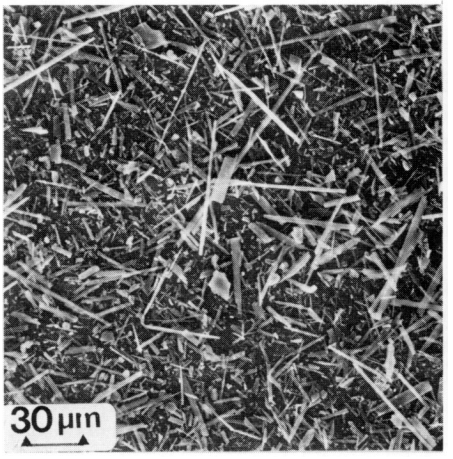
The effect of calcium was also investigated by adding a solution of $CaCl_2$ (0.8 *mM* and 30 *mM*) to the buffered saline before the RBC and the dust suspensions were mixed.

Results

SEM photographs of the different dusts are shown in Figure 1. The maximal diameter of the irregular particles of the nonfibrous minerals rarely exceeded 7 μm. The length distributions of the fibrous samples varied, CaSi A presenting the highest aspect ratio. CaSi B and CaSi E had approximately similar aspect ratios, but in general only half the ratio of CaSi A. The detailed data are given in Tables 1-3. X-ray diffraction analysis revealed unreacted quartz and calcite impurities in the synthetic compounds, the quartz content ranging from 2 to 10% (Table 4). The specific surface areas of the dust samples are shown in Table 5.

The hemolytic activity of calcium silicates is higher for the synthetic compounds than for the natural wollastonite. No differences were found within the group of synthetic compounds or within the group of natural wollastonites (Fig. 2).

Ultrasonication of the dust samples for 5 or 10 min increased the hemolytic activity for all synthetic compounds (Fig. 3). A two- to three-fold increase was observed for CaSi E at a concentration of 0.5 mg/mL. Ultrasonication had no effect on the natural CaSi B or on the positive or negative control dusts.



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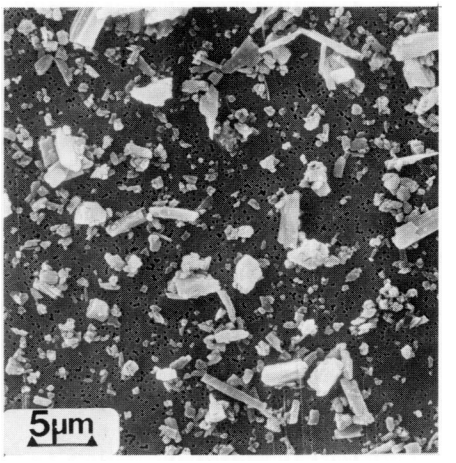

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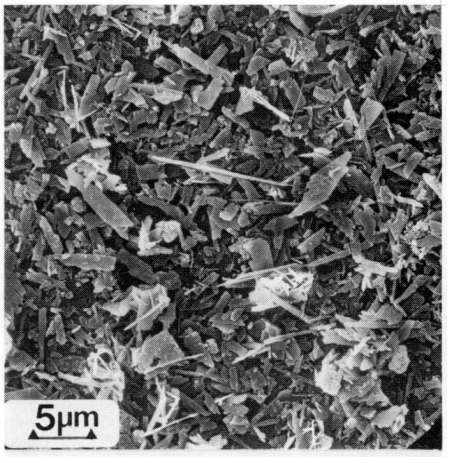

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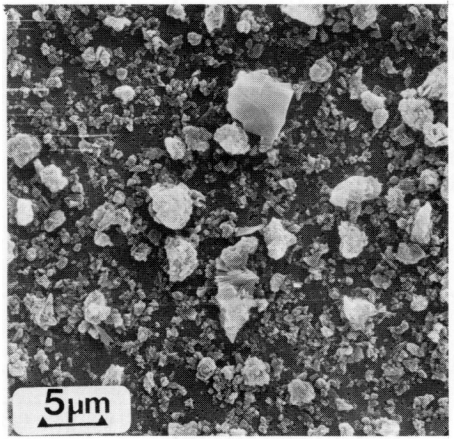

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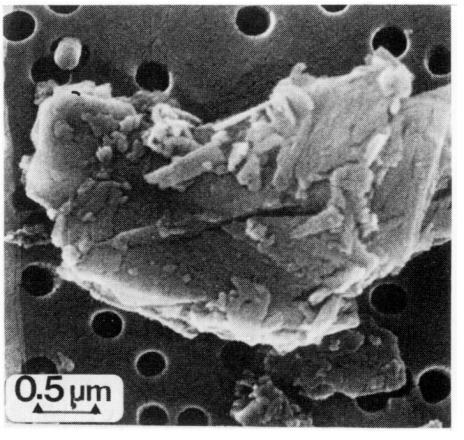

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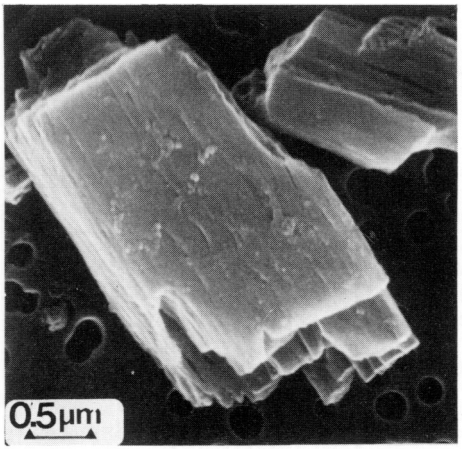

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FIGURE 1. Scanning electron micrographs of calcium silicates: (*a*) natural fibrous wollastonite (Casi *A*) (× 235); (*b*) natural wollastonite with few fibers (CaSi B) (× 1674); (*c*) fibrous synthetic tobermorite (CaSi E) (× 1564); (*d*) nonfibrous synthetic tobermorite (CaSi D) (× 1564);(e)CaSi D(x16008);(f)CaSi B(x15640).

In all experiments higher dust concentrations increased the hemolysis (Fig. 3). Departure from linearity was negligible whether the minerals were ultrasonicated or not. UICC chrysotile- and TiO_2-induced hemolysis was unaffected by dust concentration.

Table 1. Size distribution of CaSi A.

Length range, μm	Distribution of particle diameter 0-1 μm	1-2 μm	2-3 μm	3-4 μm
2-4	16	2		
4-10	26	7	1	
10-30	11	8	8	3
30-100	3	7	6	2
100-200			1	1

Table 2. Size distribution of CaSi B.

Length range, μm	Distribution of particle diameter 0-0.2 μm	0.2-0.4 μm	0.4-0.6 μm	0.6-0.8 μm	0.8-1.0 μm	1.0-1.2 μm	1.2-1.8 μm
0-1	11	3					
1-2	20	30	5	1			
2-3	10	18	9	3	1		
3-4	4	9	13	10	5	3	
4-5		3	2	2	5	2	
5-6		2	1	2	1	2	2
6-7		1	1	2	3	2	
≧7		3	1		1		6

Table 3. Size distribution of CaSi E.

Length, range, μm	Distribution of particle diameter 0-0.2 μm	0.2-0.4 μm	0.4-0.6 μm	0.6-0.8 μm	0.8-1.2 μm
0-1	46	7			
1-2	38	38	8		
2-3	9	19	9	3	
3-4	1	7	2	2	1
4-5		2			1
5-6		3	1		1

Table 4. X-ray characteristics and main chemical formulas of calcium silicates.

Sample	Chemical formula of main component	SiO_2, %	Fibrous character
CaSi A (fibrous natural wollastonite)	$CaSiO_3$	–	+ + +
CaSi B (natural wollastonite with few fibers)	$CaSiO_3$	2 ± 1	+
CaSi C (nonfibrous synthetic wollastonite)	$CaSiO_3$	9 ± 1	–
CaSi D (nonfibrous synthetic tobermorite)	$Ca_5Si_6O_{17} \cdot 2.5\ H_2O$	10 ± 2	–
CaSi E (fibrous synthetic tobermorite)	$Ca_5Si_6O_{17} \cdot 2.5\ H_2O$ $Ca_5Si_6O_{17}(OH)_2$	2 ± 1	+

Reduced hemolytic ability was not found for the CaSi C and CaSi D at pH 7.2 and 6.2 when 10 *μM* EDTA was added compared to a 30-35% reduction for UICC chrysotile B. Neither did 4 *mM* EDTA inhibit hemolysis of CaSi C at pH 7.4 when the dust was treated for 1 hr before incubation. EGTA concentrations up to 32 *mM* did not inhibit the hemolysis of CaSi B, C, D and E.

$CaCl_2$ at a level of 0.8 *mM* did not affect hemolytic activity of CaSi C. There was an increase in hemolytic activity of CaSi D and E at 30 *mM* $CaCl_2$, whereas CaSi C-induced hemolysis was not affected (Table 6).

Table 5. Specific surface area of calcium silicates.

	Specific surface area, m^2/g
CaSi A	5.1 ± 0.2
CaSi B	5.3 ± 0.2
CaSi C	18.6 ± 0.5
CaSi D[a]	50.1 ± 0.5
CaSi E[b]	22.9 ± 0.5

[a]Crystal water: 4.4%.
[b]Crystal water: 9.2%.

Table 6. Hemolysis of mineral dusts in the presence of 30 *mM* $CaCl_2$.

Sample	Hemolysis, % No Ca added	30 *mM* $CaCl_2$ added
CaSi C	39 ± 2	44 ± 5
CaSi D	28 ± 6	61 ± 2
CaSi E	56 ± 4	80 ± 6
Fragility control	1 ± 0.1	1 ± 0.1

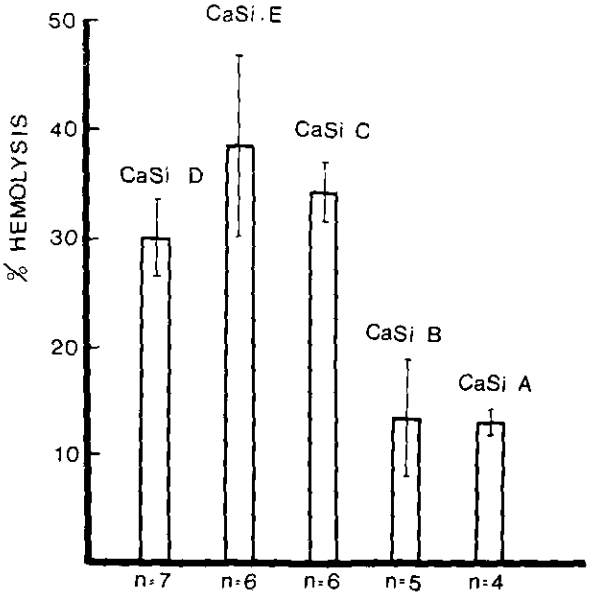

FIGURE 2. Hemolysis induced by the different CaSi (mean ± SD).

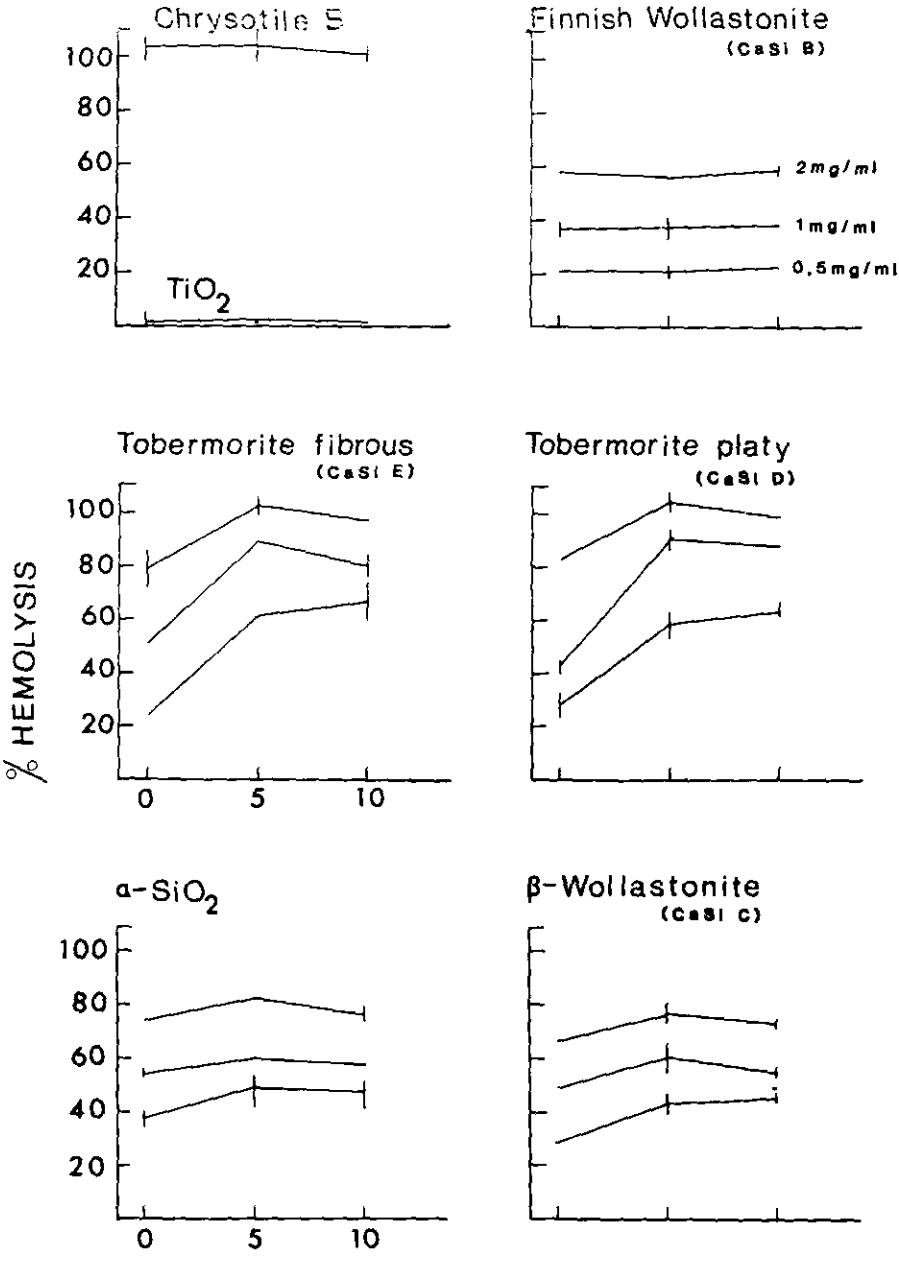

FIGURE 3. Variation in hemolysis due to ultrasonication of the minerals and due to different dust concentrations.

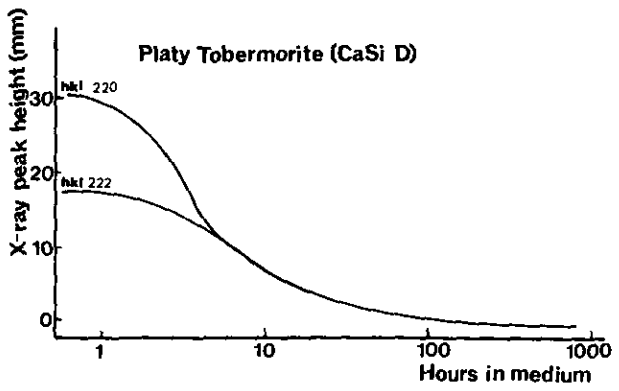

FIGURE 4. X-ray intensities of two main peaks of Tris buffer-leached CaSi D related to time in solution.

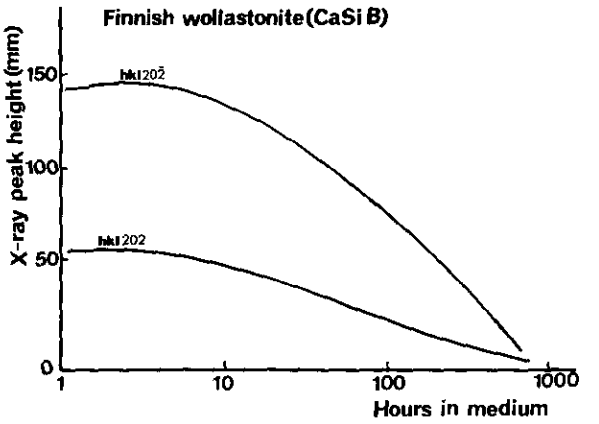

FIGURE 5. X-ray intensities of two main peaks of Tris buffer-leached CaSi B related to time in solution.

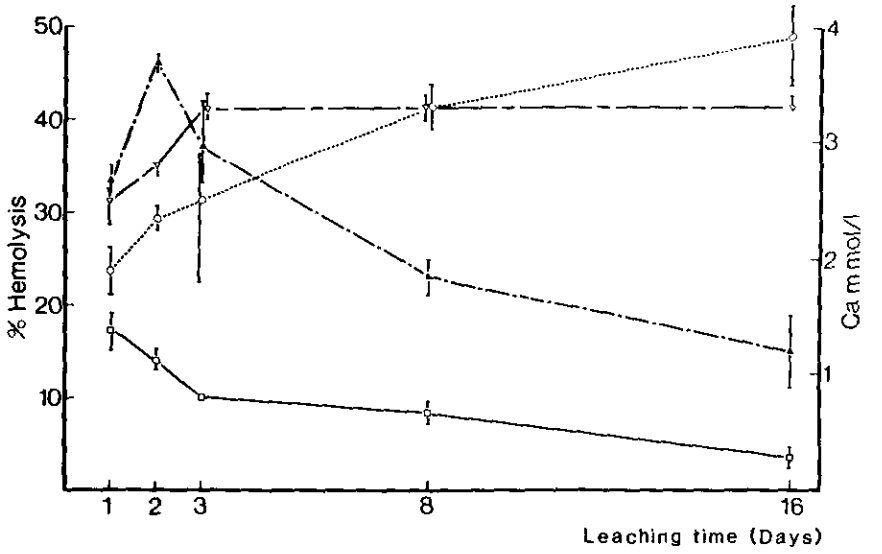

FIGURE 6. Amount of calcium released in Tris-buffered saline for (○) CaSi B and (△) CaSi D and hemolysis induced by leached (□) CaSi B and (▲) CaSi D as a function of leaching time.

Crystallography revealed that the main X-ray peaks from CaSi D almost disappeared after 1-2 days in solution, whereas CaSi B was somewhat more resistant. After 16 days the crystalline structure of CaSi B was completely broken down (Figs. 4 and 5).

Figure 6 shows a steady increase in the total amount of calcium released into the Tris-buffered saline from CaSi B and D. The hemolytic activity in leached samples decreased correspondingly. SEM photographs did not show definite morphological changes of the dust samples in solution. The amount of calcium released was low for all CaSi, CaSi D presenting the highest value (3.9 *mM* after 16 days in solution at 37°C).

SEM examination of the RBC showed that the nature of the membrane damage was similar for all CaSi examined. The initial damage was limited to a few RBC with mineral dust adherent on the surfaces. Some RBC did not show gross abnormalities, although they were covered by CaSi. The main deformities appeared as invaginations of the membranes (Fig. 7). Major cellular abnormalities were present after 5 min.

However, intact cells were still found after 60 min, also for the most hemolytic samples. The chrysotile fibers covered more of the total surface of the RBC. They were also agglutinated to a larger extent by the chrysotile fibers. Heavy deposits of TiO_2 on the RBC gave only minor change in surface shape after 60 min.

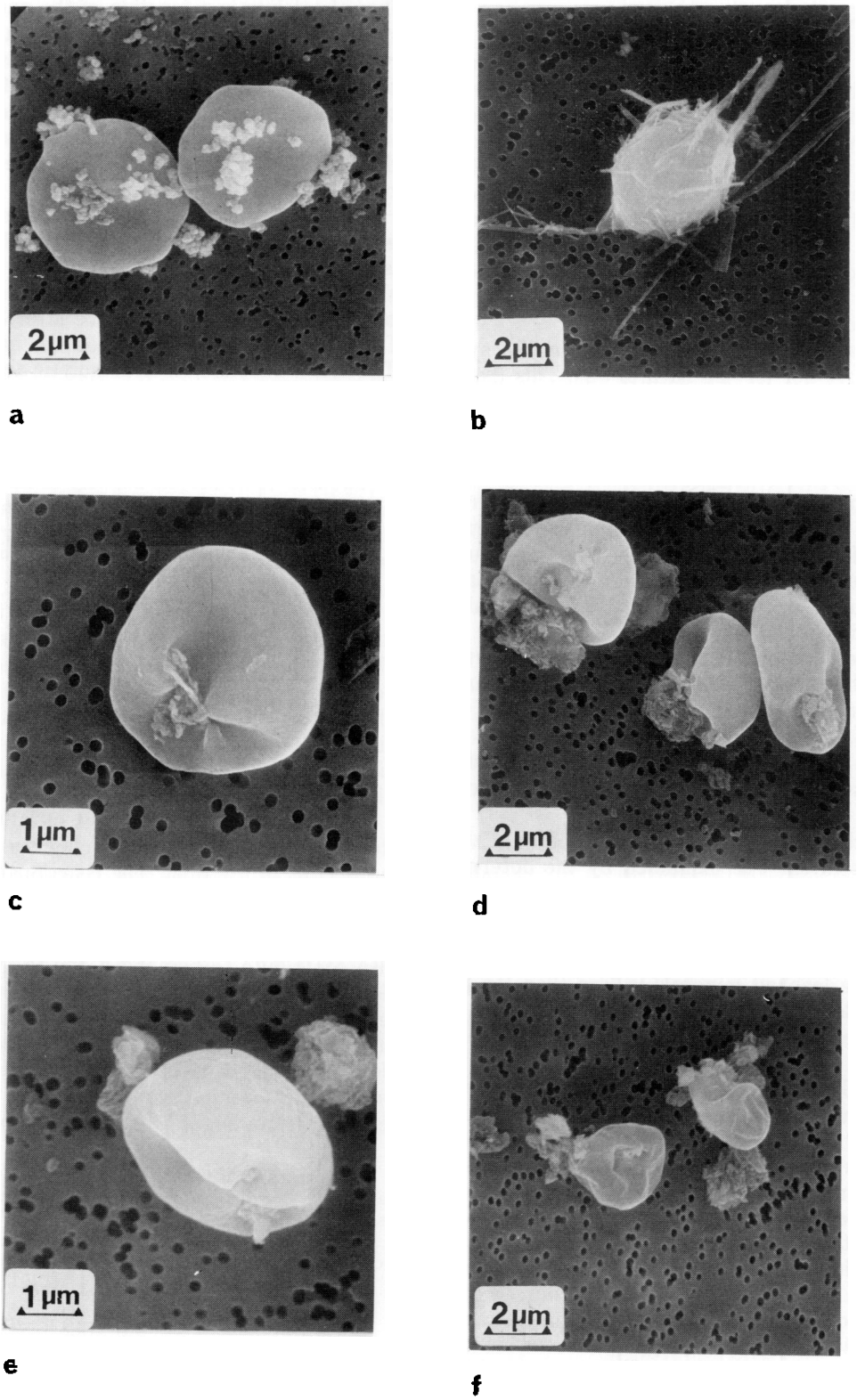

FIGURE 7. Scanning electron micrographs of RBC and mineral samples after different incubation times: (*a*) TiO_2 (60 min); (*b*) UICC chrysotile B (5 min); (*c*) CaSi E (5 min); (*d, e*) CaSi C (5 min); (*f*) CaSi C (60 min).

Discussion

Hemolytic activity is related to the specific surface area of the CaSi. The specific surface area values are notably high for the synthetic compounds, whereas they are lower for the natural CaSi, approximating those of respirable chrysotile samples (*10*). However, the value for CaSi D was disproportionately high. For this mineral the surface area determination may have been affected by the liberation of crystal water. Particle size (i.e., surface area) is thus an important factor for the hemolytic activity of these dusts.

Ultrasonication increased hemolysis for the synthetic CaSi, but not for the natural one. This treatment may disperse mineral agglomerates and even disrupt particles. The surface area available for the reaction with the RBC would thus increase. Based on this assumption, ultrasonication disintegrates synthetic compounds more easily than the natural wollastonite. Variation in hemolysis between parallels of individual samples was lowest when ultrasonication was omitted. This may indicate that ultrasonication causes uneven disintegration of the minerals in solution.

Mineral morphology does not influence hemolytic activity, as shown by the comparison of acicular and fibrous minerals within the groups of synthetic or natural CaSi. Neither do the quartz impurities of the minerals increase hemolysis compared to minerals without quartz within the same group (Table 4 and Fig. 2).

In the leaching experiments, the durability of the minerals in solution was examined by the determination of calcium release and by changes in the mineral structure. Small amounts of calcium are released from the minerals to the saline. Hemolysis induced by the leached minerals is less than for the parent samples.

A possible role of calcium in the mechanism of CaSi-induced hemolysis was not confirmed by EDTA or EGTA treatment. EDTA has been shown to inhibit chrysotile hemolysis, probably by complexing Mg^{2+} on the surface layer (*11*). The chelator treatment is likely to inactivate calcium released from the minerals to the solution, but we do not know whether this treatment inactivates high calcium concentrations on the surface of the particles responsible for local reactions with the RBC membranes. RBC membrane damage and hemolysis may be related to abnormal influx of calcium into the cells, as shown in the RBC of sickle cell anemia (*12*). The calcium concentration in RBC cytosol is normally less than 0.1 μM (*13*). An abnormal influx of calcium may occur secondary to mineral-induced mechanical damage of the RBC. The steady-state influx of calcium is a function of the extracellular calcium concentration (*14*). These mechanisms may be responsible for the increased hemolysis of the synthetic CaSi D and E when 30 mM $CaCl_2$ is added to the RBC suspension. Increased intracellular calcium may thus be a contributing mechanism for CaSi-induced hemolysis, but the definite role of calcium remains to be established.

The X-ray diffraction patterns from the two minerals indicate that rupture of crystalline structures is already initiated at the second day in solution, as shown by reduction in the peak intensities followed by the complete disappearance after 3 days for the CaSi D, and after 16 days for the natural CaSi B. The simultaneous loss of hemolytic activity, notably from the third day, indicates that alteration in crystalline structure may be responsible for decreased hemolysis.

The loss of hemolytic activity of the UICC chrysotile B dust samples during the experimental period may be due to alteration in mineralogical characteristics by permanent storage at 37°C to remove humidity.

The SEM microphotographs of the cells and dusts visualize the adherence of dust particles to the RBC membranes and agglomeration of cells and cellular debris. This may be an initial event, as there is no substantial change in the amount of particles adhered to individual cells during the incubation period. Morphological similarities to typical sickle cells were not seen.

In conclusion, the hemolysis of RBC due to CaSi dust exposure depends on chemical and physical properties of the dust. The reactions with the RBC membranes are related to the surface area of the minerals. Crystalline SiO_2 content is not likely to contribute to the hemolytic activity. Calcium may also contribute to hemolysis due to high concentrations at the interphase between RBC membranes and minerals, and by a postulated increased influx of calcium into the RBC. Another line of investigation is the follow-up of the crystallographic changes during the leaching experiments, in which the released calcium might be a secondary phenomenon.

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